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HPLC Method for Quantification of Ornidazole in Human Plasma

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A sensitive and rapid reverse phase HPLC method was developed to quantify plasma levels of ornidazole, to evaluate a comparative bioavailability study. The drug and internal standard were purified from plasma samples by liquid-liquid extraction using dichloromethane. The HPLC separation was performed on BDS hypersil C_{18} column (250 × 4.6 mm, 5 µ), using 0.05 M disodium hydrogen phosphate (pH 4.8) and acetonitrile (60:40 v/v) with a flow rate of 1 mL/min and UV detection at 313 nm. Standard curve covering 50 ng-12 µg/mL concentration range, was linear (r^2 = 0.9997). The precision and accuracy of ornidazole was 3.52 > % RSD and 3.6 > % RME in intra-day and inter-day analysis after four quality control very low, low, medium and high (QCVL, QCL, QCM and QCH) samples. The bio-study was carried out in 12 healthy human volunteers according to a single dose, two-sequence, cross over randomized design. The 90 % confidence interval for the ratio of the logarithmic transformed $AUC_{0-\infty}$ (0.83-1.02) and Cmax (0.93-1.03) were within the bioequivalence limit of 0.80-1.25.

Key Words: Ornidazole, HPLC, Bioequivalence, Human plasma.

INTRODUCTION

Chemically, ornidazole is 1-chloro-3-(2-methyl-5-nitroimidazol-1-yl)-propan-2-ol, with antiprotozoal and antibacterial properties against anaerobic bacteria. Its antimicrobial activity is due to the reduction of the nitro group to a more reactive amine that attacks microbial DNA, inhibiting further synthesis and causing degradation of existing DNA[1]. Used in the treatment of severe hepatic and intestinal amoebiasis, giardiasis, trichomoniasis of urogenital tract and bacterial vaginosis. It is also used in the treatment and prophylaxis of susceptible anaerobic infections in dental and gastrointestinal surgery[2]. Ornidazole is readily absorbed from the gastro-intestinal tract

and peak plasma concentrations of about 30 µg/mL have been achieved within 2 h of a single dose of 1.5 g, falling to about 9 µg/mL after 24 h and 2.5 µg/mL after 48 h. The plasma elimination half-life of ornidazole is 12 to 14 h. Less than 15 % is bound to plasma proteins. It is widely distributed in body tissues and fluids, including the cerebrospinal fluid. Ornidazole is metabolized in the liver and is excreted in the urine, mainly as conjugates and metabolites and to a lesser extent in the faeces; 85 % of a single oral dose has been reported to be eliminated within 5 d, 63 % in the urine and 22 % in the faeces[3]. With ornidazole, disulfiram like reaction has not been reported on consumption of alcohol[4]. Bioavailability issues have been an increasing concern to drug regulatory authorities for assessment of the safety and efficacy of drug products[5]. As the number of synonym drug products increase, special attention in bioavailability issues becomes a major concern. Local drug regulatory authorities have therefore, issued guidelines to ensure adequate bioavailability studies in new drug applications for synonym drugs[6]. The purpose of this study was to compare the bioavailability of generic ornidazole 500 mg tablet (ORGYL), manufactured by Kusum Healthcare, an Indian Pharmaceutical Company, with that of the innovator TIBERAL® (F. Hoffmann - La Roche Ltd, Switzerland). To evaluate this, a suitable HPLC method was required. HPLC methods utilize complicated extraction procedures[7,8] involving protein precipitation or solid-phase extraction[9,10] in which some methods are not applicable for pharmacokinetic and bioequivalence study of tablets in adults[11-14]. So, the main aim was, to establish a rapid and sensitive HPLC method for quantification of ornidazole concentrations in human plasma with a simple liquid-liquid extraction which produced excellent recovery.

EXPERIMENTAL

A gradient HPLC system of KNAUER (Germany) consisting of a solvent delivery pump (K-1000), degasser Manager K-5000, auto-sampler-3800 and a UV-Visible detector (K-2501) and software ezchrom elite was utilized for determining drug concentrations and data analysis. Pure drugs of ornidazole and internal standard (tinidazole) were obtained from sigma-Aldrich (US). All reagents and solutions were HPLC or analytical grade. Plasma samples were stored in -20 °C deep freezer. Distilled water was prepared by Milli-Q (A-10, Elix-Academic, France) with 0.22 µm filter. Automatic laboratory centrifuge (RC-108, Remi equipments Ltd., Mumbai, India) was used to centrifuge the plasma samples.

Chromatogram separation was done using a BDS hypersil C_{18}, (250 × 4.6 mm, 5 µ, UK) column with a loop size of 100 µL. The mobile phase consisted of 0.05 M disodium hydrogen phosphate (pH 4.8) and acetonitrile (60:40 v/v) with a flow rate of 1 mL/min and was filtered through 0.45 µm

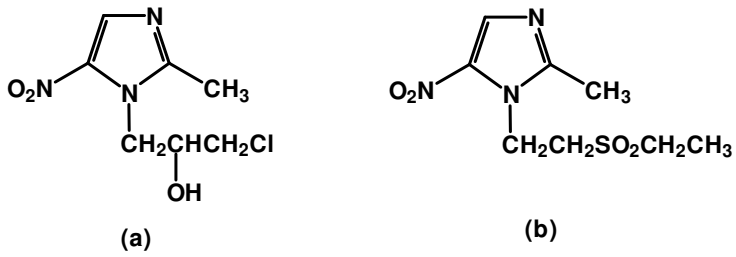
filters. The samples were injected using micro syringes (Hamilton Bonaduz AG, Switzerland) and was monitored using UV detection at 313 nm. Quantification was done by calculating the peak area ratios of the drug to the internal standard

Sample preparation: 1 mL of plasma was taken in a stopper test tube. To this 0.1 mL of tinidazole (I.S., 200 ng/mL) was added and mixed for 1 min. In this mixture, 7 mL of dichloromethane was added and vortex-mixed for 10 min. It was then centrifuged for 5 min at 3000 rpm. 6 mL of organic layer was removed in a separate tube and evaporated under nitrogen atmosphere at 40-50 °C. The residue was reconstituted with 200 µL of mobile phase and was injected into the HPLC system.

Calibration procedure: A stock solution of ornidazole was prepared by dissolving 100 mg in 100 mL of methanol. Calibration samples of ornidazole were prepared in blank plasma at various concentrations of 50 ng, 100 ng, 400 ng, 1 µg, 4 µg, 8 µg and 12 µg/mL. The calibration curve was obtained by plotting peak area ratios of ornidazole to internal standard *versus* ornidazole concentrations. Plasma drug concentrations in volunteer samples were calculated by determination of the peak area ratio of ornidazole to internal standard and comparing the response with those of the standard curve by substituting the values in the regression equation.

Bio-analytical method validation: Quality control samples of QCVL-50, QCL-150, QCM-5000 and QCH-9000 ng/mL were analyzed in six replicates on the same day to determine the intra-day precision and accuracy and on each of five separate days to determine inter-day precision and accuracy. The quality control samples were analyzed for freeze-thaw stability when stored at -20 °C for 3 months. The absolute recovery was determined by comparing the peak areas of quality control samples to that of the raw samples prepared in mobile phase, of same concentration. The limit of detection (LOD) was calculated from the lowest concentration which gave signal-to-noise ratio of 3. The limit of detection (LOQ) was the lowest concentration which was in the linearity range[15].

***in vivo* Study design:** Approval from Drugs Control General of India (DCGI) and Institutional Ethical Committee of Jadavpur University was obtained prior to the start of the study. 12 Indian male volunteers aged between 18 and 25 years and with body mass index between 20 and 24 were included in a randomized, single dose, two phase, two sequence, cross-over study with a 1 week washout period. A total of 15 blood samples were collected at 0 h (before drug administration) and at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 10.0 12.0 and 24.0 h (after drug administration) in test tubes with heparin. Collected blood samples were centrifuged and plasma was separated and stored frozen[16,17] at -20 ºC.



Chemical Structures of (a) ornidazole and (b) tinidazole

Pharmacokinetic and statistical analysis: The pharmacokinetic parameters were calculated by the standard non-compartmental method. Both maximum plasma concentration (Cmax) and time to peak plasma concentration (tmax) were obtained from the data. The elimination half-life ($t_{½}$) was calculated as 0.693/Kel. Kel was calculated as the slope of the linear regression line of natural log-transformed plasma concentrations. The last seven quantifiable levels were used to determine Kel. The area under the plasma concentration-time curve (AUC_{0-t}) was calculated from the measured levels, from time zero to the time of last quantifiable level, by the linear trapezoidal rule. ($AUC_{0-\infty}$) was calculated according to the following formula: $AUC_{0-\infty} = AUC_{0-t} + C_{last}/Kel$, where C_{last} is the last quantifiable plasma level. Analysis of variance was performed on the pharmacokinetic parameters Cmax, $AUC_{0-\infty}$ using general linear model (GLM) procedures. The 90 % confidence interval of the test/reference ratios for Cmax, AUC_{0-24} and $AUC_{0-\infty}$ (log transformed) were determined. Bioequivalence between the two formulations was concluded when 90 % confidence interval for the pharmacokinetic parameters of the two products were found[18,19] within the acceptable range of 80-125 %.

RESULTS AND DISCUSSION

Sensitivity and linearity: The limit of quantification (LOQ) for ornidazole in plasma was 50 ng/mL and the lower limit of detection (LOD) was 20 ng/mL. The relationship between concentration and peak area was found to be linear within the range of 50 ng to 12 μg/mL ($r^2 = 0.9997$). An internal standard (tinidazole) was used in the study and the log-log model constructed between peak height and ornidazole concentration proved to be linear over a range of concentration measured and with less per cent deviation for each point.

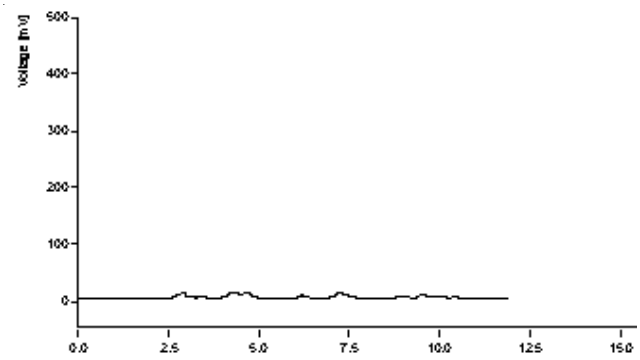
Precision and accuracy: Ornidazole showed precision (0.36-3.31 % RSD) and accuracy (0.69-3.4 % RME) in intra-day and precision (0.37-3.52 % RSD) and accuracy (0.76-3.6 % RME) in inter-day analysis (Table-1).

TABLE-1
INTRA-DAY AND INTER-DAY, PRECISION AND
ACCURACY OF ORNIDAZOLE (n = 6)

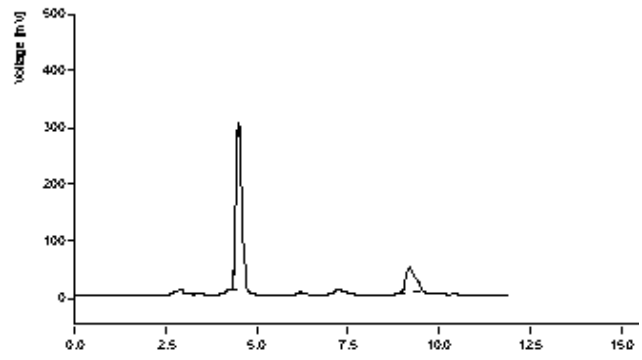
QC sample (ng/mL)	Intra-day variation			Inter-day variation		
	Mean ± SD	RSD (%)	RME (%)	Mean ± SD	RSD (%)	RME (%)
50	48.3 ± 1.6	3.31	3.40	48.2 ± 1.7	3.52	3.60
150	147.6 ± 1.8	1.21	1.60	146.4 ± 1.6	1.09	2.40
5000	4954.8 ± 26.6	0.53	0.90	4948.6 ± 28.5	0.57	1.02
9000	8937.7 ± 32.5	0.36	0.69	8931.5 ± 33.2	0.37	0.76

SD = Standard deviation; RSD = Relative standard deviation ;
RME = Relative mean error.

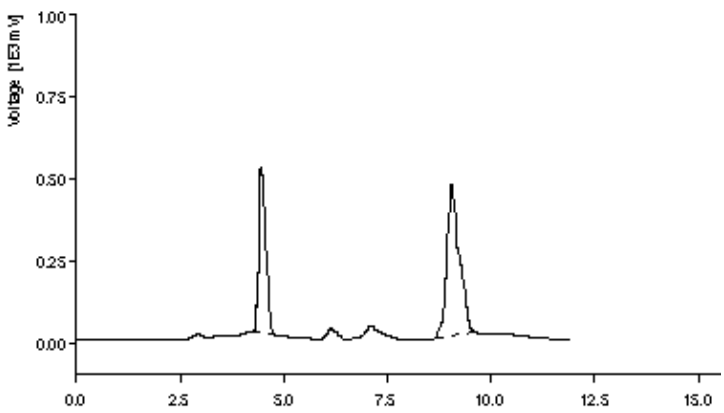
Resolution and recovery: In this HPLC method, the retention time for ornidazole was 9.2 min and tinidazole (IS) was 4.5 min (Fig. 1C) which shows good resolution of peaks. Throughout the stability tests, ornidazole proved stable in biological samples for at least two freeze and thaw cycles with a final mean recovery of 98.43 % and the absolute recovery was between 97.08-99.29 % with RSD < 1.80 % (Table-2).



(A)



(B)



(C)

Fig. 1. HPLC Chromatograms of ornidazole (A) Blank plasma (B) Spiked plasma sample of 50 ng/mL (LOQ) concentration of ornidazole (C) Volunteer sample containing peaks of tinidazole (IS)-4.5 min and ornidazole - 9.2 min

TABLE-2
ABSOLUTE RECOVERY OF ORNIDAZOLE (n = 5)

Concentration (ng/mL)	Obtained mean ± SD	Recovery (%)	RSD (%)
50	48.54 ± 0.9	97.08	1.80
150	147.80 ± 1.2	98.53	0.81
5000	4952.70 ± 26.7	99.05	0.53
9000	8936.30 ± 35.2	99.29	0.39

Pharmacokinetics and bioequivalence: Cmax levels were observed after 2.33 ± 0.47 h (test) and 2.37 ± 0.35 h (reference). The Cmax and $AUC_{0-\infty}$ of test and reference were 10.68 ± 0.97 *vs.* 10.86 ± 1.24 μg/mL and 151.61 ± 35.58 *vs.* 165.69 ± 23.85 μg/mL h. The mean $t_{½}$ for test and reference were 14.94 ± 3.64 h and 14.96 ± 4.59 h, respectively (Table-1). The elimination half life of ornidazole was in the range 12-14 h. Thus the washout period of one week was sufficient, due to the fact that no sample prior to administration in phase 2 showed ornidazole levels. Average concentration *versus* time curves after administration of reference and test products to healthy volunteers are shown in Fig. 2. The limits of the 90 % confidential interval for the ratios of AUC_{0-t}, $AUC_{0-\infty}$ and Cmax for their log transformed data were within 0.80 to 1.25 (Table-3).

Since, the liquid-liquid extraction method showed excellent recovery of ornidazole from plasma, it proved both formulations were equal in terms of rate and extent of absorption. The aim of the bioequivalence trials is to assure interchangeability between an innovator and a generic formula in

TABLE-3
PHARMACOKINETIC PARAMETERS

Parameter	Test	Reference	90 % CI
AUC_{0-t} (µg/mL h)	101.950 ± 13.000	112.270 ± 19.230	84.90-98.63
$AUC_{0-\infty}$ (µg/mL h)	151.610 ± 35.580	165.690 ± 23.850	83.54-102.82
Cmax (µg/mL)	10.680 ± 0.970	10.860 ± 1.240	93.51-103.90
Tmax (h)	2.330 ± 0.470	2.370 ± 0.350	–
$T_{½}$	14.940 ± 3.640	14.960 ± 4.590	–
Kel	0.0490 ± 0.011	0.051 ± 0.017	–

AUC_{0-t}, $AUC_{0-\infty}$ = Area under plasma concentration time curve and at infinity time; Cmax = Maximum plasma concentration; Tmax = Time to reach maximum plasma concentration; $t_{½}$ = Elimination half life; Kel = apparent elimination rate constant.

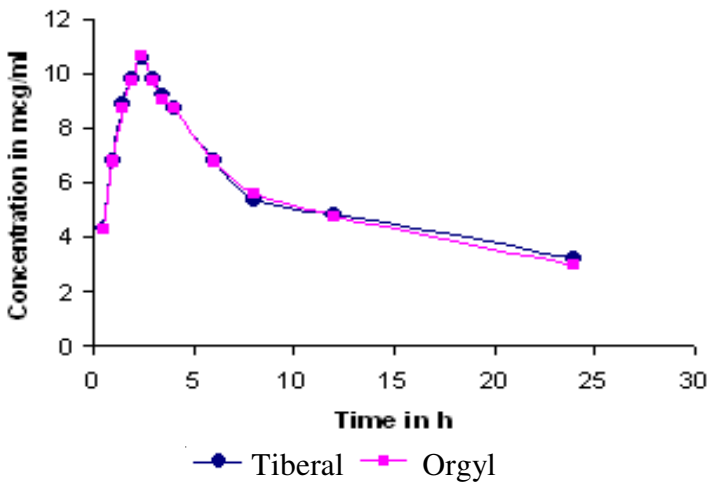


Fig. 2. Plasma concentration *vs.* time profile of ornidazole

terms of efficacy and safety. When a pharmacological effect is difficult to measure, the plasma levels of drug may be used as an indirect indicator of clinical activity. Therefore, the ornidazole plasma levels obtained in this study suggest an equal clinical efficacy of the two brands tested and provide pharmacokinetic data from an Indian population when evaluated by this HPLC method. Consequently, bioequivalence between the two ornidazole preparations can be concluded.

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